Kinetic Mechanism of Histidinol Dehydrogenase: Histidinol Binding and Exchange Reactions†

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ABSTRACT: *Salmonella typhimurium* histidinol dehydrogenase produces histidine from the amino alcohol histidinol by two sequential NAD-linked oxidations which form and oxidize a stable enzyme-bound histidinaldehyde intermediate. The enzyme was found to catalyze the exchange of ^3H between histidinol and [4(*R*)-^{3}H]NADH and between NAD and [4(*S*)-^{3}H]NADH. The latter reaction proceeded at rates greater than k_{cat} for the net reaction and was about 3-fold faster than the former. Histidine did not support an NAD/NADH exchange, demonstrating kinetic irreversibility in the second half-reaction. Specific activity measurements on [^{3}H]histidinol produced during the histidinol/NADH exchange reaction showed that only a single hydrogen was exchanged between the two reactants, demonstrating that under the conditions employed this exchange reaction arises only from the reversal of the alcohol dehydrogenase step and not the aldehyde dehydrogenase reaction. The kinetics of the NAD/NADH exchange reaction demonstrated a hyperbolic dependence on the concentration of NAD and NADH when the two were present in a 1:2 molar ratio. The histidinol/NADH exchange showed severe inhibition by high NAD and NADH under the same conditions, indicating that histidinol cannot dissociate directly from the ternary enzyme–NAD–histidinol complex; in other words, the binding of substrate is ordered with histidinol leading. Binding studies indicated that [^{3}H]histidinol bound to 1.7 sites on the dimeric enzyme (0.85 site/monomer) with a K_D of 10 μM. No binding of [^{3}H]NAD or [^{3}H]NADH was detected. The nucleotides could, however, displace histidinol dehydrogenase from Cibacron Blue–agarose. We conclude that the first half-reaction of histidinol dehydrogenase follows an ordered mechanism in which L-histidinol leads, in confirmation of the mechanism proposed from steady-state kinetics [Burger, E., & Gorisch, H. (1981) *Eur. J. Biochem. 116*, 137–142].

Salmonella typhimurium histidinol dehydrogenase catalyzes the four-electron NAD-linked oxidation of L-histidinol to L-histidine in a two-step reaction, with both steps occurring at a single active site (Adams, 1955). It is clear that a very tightly associated form of the labile compound histidinaldehyde is an intermediate between the two reactions (Adams, 1955). Although the chemical nature of the enzyme–histidinaldehyde intermediate is not clear, it can be isolated in stable form (Gorisch & Holke, 1985).

It has been proposed (Gorisch, 1979; Burger & Gorisch, 1981) that histidinol dehydrogenase follows a bi-uni-uni-bi ping-pong mechanism in which the initial enzyme–histidinol–NAD complex is formed by the ordered binding of first histidinol and then NAD. The proposed order is nearly unique among NAD-linked dehydrogenases, being shared only by UDP-glucose dehydrogenase (Ordman & Kirkwood, 1977) and yeast aldehyde dehydrogenase (Bradbury & Jakoby, 1971). The order is of interest in that NAD binding to most dehydrogenases triggers a conformational change that permits subsequent substrate binding [see Grau (1982)].

In the present study the kinetic mechanism of the first half-reaction was investigated by using exchange reactions and binding studies. We confirm the proposed kinetic mechanism.

Experimental Procedures

Histidinol dehydrogenase was prepared according to the method of Yourno and Ino (1968) from cells of *S. typhimurium hisO1242* (obtained from John Roth, University of Utah) grown by high-density fermentation (Grubmeyer & Gray, 1986). The enzyme was stored as a crystalline suspension. Before use, the required volume of the suspension was centrifuged for 2 min in an Eppendorf microfuge and the protein pellet then dissolved in the desired buffer. Protein determination used the known extinction coefficient $\epsilon^{0.1\%}_{280nm}$ of 0.478 (Yourno, 1968). The subunit molecular weight of 45823 from protein sequencing (Kohno & Gray, 1981) was used to calculate subunit molarity in solutions of the stable dimer (Loper, 1968; Burger et al., 1979). The standard continuous assay of enzyme activity was performed as described (Grubmeyer & Gray, 1986) and employed 1-mL mixtures containing 50 μmol of glycine–NaOH, 2 μmol of histidinol, 0.5 μmol of $MnCl_2$, and 10 μmol of NAD, brought to pH 9.2 with NaOH.

Exchange reactions were conducted in 1-mL assay mixtures containing 50 μmol of glycine–NaOH, 0.5 μmol of $MnCl_2$, 2 μmol of histidinol, 3×10^5 cpm of [4-^{3}H]NADH of appropriate stereochemistry, and nonlabeled NAD and NADH as indicated. The pH of exchange mixtures was adjusted to 9.2 with NaOH, and the reaction mixtures were allowed to come to 30 °C in a thermostated block heater. Enzyme (5–10 μg) was added to start the reaction. The reaction was terminated at the indicated time by application of a 100-μL sample to a column of (diethylaminoethyl)cellulose (DEAE-cellulose).

The histidinol, NAD, and NADH were separated following the general scheme of Davies et al. (1972). Columns (0.5 × 4 cm) of (diethylaminoethyl)cellulose were preequilibrated with 50 mM (Tris-HCl), pH 8.0. After sample application, the column was washed with one 0.9-mL and four 1-mL portions of the same buffer, and five 1-mL fractions were collected. The column was then further eluted with three 1-mL portions of 200 mM NaCl in 50 mM Tris-HCl, pH 8.0. Fraction 1 contained histidinol, fractions 3 and 4 contained

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NAD, and fractions 6 and 7 contained NADH. Radioactivity was measured by liquid scintillation counting on 300-μL samples in 3 mL of Scinti-Verse II (Fisher) or Liquiscint (National Diagnostics). The overall recovery of radioactivity from the column was 90–100%.

In some experiments, the histidinol concentration in column fractions was quantitated enzymatically by using the histidinol dehydrogenase reaction as suggested by Gorisch (1979). To 0.9 mL of standard assay medium (minus histidinol) in a 1-mL quartz cuvette was added 0.1 mL of sample from column eluants, and after a stable base line absorbance at 340 nm was achieved, 2 μL of a solution containing 20 μg of histidinol dehydrogenase was added. After a short incubation the stable absorbance was used to calculate the histidinol concentration.

The extent of the exchange reaction was determined by multiplying the fraction of the recovered radioactivity that was present in the exchange product (histidinol or NAD) by the number of micromoles of radioactive exchange substrate (NADH) present at the start of the reaction. This number was then used to calculate rates. It is important to note that the exchanges are not true equilibrium exchanges but occur in the presence of a slow net catalytic reaction. However, the rates reported may be taken as valid measurements when the proportion of substrate and cosubstrates converted is less than about 10%.

Net catalysis under exchange conditions (Figure 1) was assayed at 30 °C in 1-mL reactions containing 50 μmol of sodium glycinate, 2 μmol of NADH, 1 μmol of NAD, 1 μmol of histidinol, and 0.5 μmol of $MnCl_2$. The pH was brought to 9.2 with NaOH. Ten micrograms of histidinol dehydrogenase was added, and at specified times 100-μL samples were quenched by addition to 900 μL of 0.1% sodium dodecyl sulfate. The absorbance at 340 nm was then determined.

Preparation of ^{3}H-labeled NADH followed the general methods of Davies et al. (1972). For [4(*R*)-^{3}H]NADH the reaction mixture, in a final volume of 1 mL, contained 58 μmol of Tris-HCl, pH 8, 0.2 μmol of NAD, 20 μmol of Na_2HAsO_4, 1 μmol of 3-phosphoglyceraldehyde (prepared from the diethyl acetal following the procedure recommended by the supplier), and 2.5×10^6 cpm of [4-^{3}H]NAD. The mixture was incubated in a quartz cuvette for several minutes at 30 °C, and the stable base line at 340 nm was recorded. Glyceraldehyde-3-phosphate dehydrogenase (1.5 μg) [an *S*-specific enzyme; see You (1982)] was added to start the reaction, which was allowed to continue until a new stable absorbance was established. The mixture was then heated in a boiling water bath for 2 min and diluted to 10 mL with water. Product NADH was purified as described above, except that the first fraction was 10-mL volume.

The preparation of [4(*S*)-^{3}H]NADH was carried out according to a similar procedure, with *S. typhimurium* histidinol dehydrogenase (an *R*-specific enzyme; Grubmeyer and Chu, unpublished results). The reaction mixture contained 50 μmol of sodium glycinate, pH 9.2, 0.5 μmol of $MnCl_2$, 0.2 μmol of NAD, 2 μmol of histidinol, and [4-^{3}H]NAD. Histidinol dehydrogenase (20 μg) was added to start the reaction. Purification of NADH was carried out as described above.

[^{3}H]Histidinol was synthesized via the histidinol dehydrogenase catalyzed histidinol/NADH exchange reaction. Histidinol dehydrogenase (100 μg) was dissolved in 1 mL of buffer containing 35 μmol of glycine–NaOH, 0.2 μmol of [4(*R*)-^{3}H]NADH, 1.0 μmol of histidinol, 0.5 μmol of $MnCl_2$, and 0.1 μmol of NAD adjusted to pH 9.2 with NaOH. The reaction was allowed to proceed in a dry bath at 30 °C for 1 h at the end of which the reaction was quenched by placing the tube in a boiling water bath for 1 min. The entire 1-mL mixture was applied to a column (0.5 × 5 cm) of (diethylaminoethyl)cellulose equilibrated with 50 mM Tris-HCl, pH 8.0, and the effluent was collected. The column was further washed with 0.5 mL of buffer at pH 8.0 and the additional effluent collected. The concentration of ^{3}H-labeled histidinol in the 1.5 mL of product was determined by the spectrophotometric histidinol dehydrogenase assay described above. The ^{3}H was determined by counting 50-μL portions in 4-mL scintillation vials with glass holders. Purity of [^{3}H]histidinol was monitored by the separation and detection systems described by Ames and Mitchell (1952), on thin-layer plates of cellulose.

Binding of [^{3}H]histidinol, [^{3}H]NAD, and [^{3}H]NADH to histidinol dehydrogenase was investigated by using the method of Hummel and Dreyer (1962) as modified by Fersht (1975). Columns of Sephadex G-50 (0.5 × 7.5 cm) were equilibrated with 2 mL of buffer consisting of 50 mM sodium glycinate, pH 9.05, 0.5 mM $MnCl_2$, 1 mM potassium phosphate, 10^5 cpm/mL [^{32}P]P_i, and ^{3}H-labeled ligand. Enzyme, 200–400 μg, was dissolved in a 500-μL portion of the equilibration buffer, and 100 μL of this solution was applied to the column. The column was eluted with 2.6 mL of the equilibration buffer. Fractions of 1 drop each were collected and counted by liquid scintillation to 1% counting error. The ^{32}P counts were used to determine the volume of each drop, which ranged from 48 to 53 μL, and the ^{3}H counts provided a measure of the total ligand in the drop. Reproducible data required accurate counting, and glass minivial holders were suitable. Plastic (Beckman) minivial holders caused substantial and variable quenching of ^{3}H counts and were not suitable. For each experiment data like those of Figure 4 were collected, plotted, and used to calculate the quantity of ligand bound, by use of the spread sheet program Flash-calc (Visi-Corp) on an Apple IIE computer. The mean of peak and trough binding was plotted to determine K_D.

L-Histidinol, L-histidine, 3-phosphoglyceraldehyde diethyl acetal, and other biochemicals were from Sigma. [4-^{3}H]NAD was from Amersham, and [^{32}P]P_i was from New England Nuclear. NAD was a product of Boehringer. DE-52 [(diethylaminoethyl)cellulose, microcrystalline] was from Whatman. All other chemicals were of high purity and were obtained from Fisher.

RESULTS

Under conditions of high NADH and low NAD concentration, histidinol dehydrogenase did not catalyze significant net oxidation of histidinol (Figure 1). Under the same conditions, the enzyme did catalyze the incorporation of radioactivity from [4(*R*)-^{3}H]NADH into the histidinol fraction from ion-exchange chromatography of the reaction mixture. The radioactive material comigrated with authentic L-histidinol on thin-layer chromatograms, and its radioactivity was transferred to NADH when incubated with histidinol dehydrogenase and NAD. Thus histidinol dehydrogenase can catalyze the reversal of the first oxidation step under normal conditions. In addition, the enzyme catalyzed an exchange of radioactivity between NAD and [4(*S*)-^{3}H]NADH under the same reaction conditions. The [^{3}H]NAD produced by the exchange could be reduced to [^{3}H]NADH by histidinol dehydrogenase. Both exchange reactions showed linear time courses.

The properties of the exchange reactions were explored further. In work not shown here, we found that both the exchanges required the presence of enzyme, NAD, and histidinol. Histamine, a competitive inhibitor of the overall re-

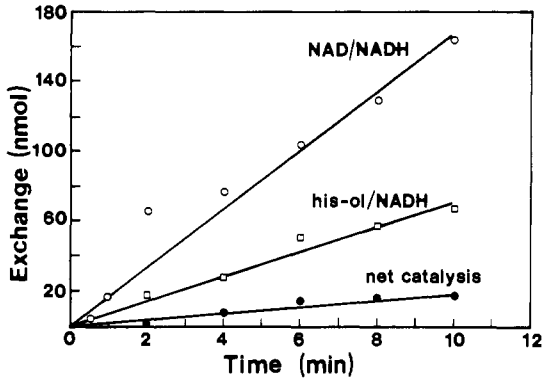


FIGURE 1: Exchange activity of histidinol dehydrogenase. Reaction mixtures contained, in a final volume of 1.0 mL, 50 mM sodium glycinate, pH 9.2, 0.5 mM $MnCl_2$, 1 mM histidinol, 1 mM NAD, 2 mM NADH, and 2.6 μg of histidinol dehydrogenase. For histidinol/NADH exchange (□) 1×10^6 cpm of [4(R)-^{3}H]NADH was added, and for NAD/NADH exchange (○) 1×10^6 cpm of [4-(S)-^{3}H]NADH was added. Net catalysis (●) was assayed as described under Experimental Procedures.

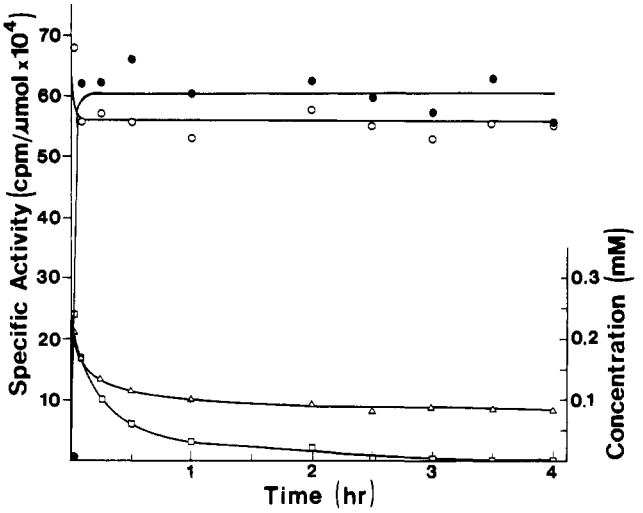


FIGURE 2: Specific activity of exchange substrates. Reaction mixtures contained, in a final volume of 1 mL, 50 mM sodium glycinate, pH 9.2, 0.5 mM $MnCl_2$, 2 mM NADH, 0.24 mM NAD, 0.2 mM histidinol, 8×10^5 cpm of [4(R)-^{3}H]NADH, and 20 μg of histidinol dehydrogenase. At the indicated times 100-μL samples were applied to DE-52 columns for analysis (Experimental Procedures). Resulting fractions were analyzed for radioactivity, histidinol, and NADH and the results used to calculate specific activity. (○) NADH specific activity; (●) histidinol specific activity; (□) NAD remaining (calculated); (△) histidinol remaining.

action vs. histidinol, also inhibited the exchange reactions. In the absence of histidinol, 5 mM histidine did not support any movement of ^{3}H from [4(R)-^{3}H]NADH into the histidinol fraction (net reversal of catalysis), nor did histidine alone support an NAD/NADH exchange, demonstrating that a kinetically irreversible step in the overall reaction mechanism occurs at or before histidine release. In separate experiments not shown here, we have also consistently failed to observe production of labeled histidinol from histidine labeled in the imidazole ring.

The kinetic scheme for the enzyme suggests two ways in which the histidinol/NADH exchange reaction could occur, either by reversal of the alcohol oxidation step alone or by reversal of both the aldehyde and alcohol oxidation steps. This subject was investigated by taking specific activity measurements during the course of the reaction (Figure 2). As exchange proceeded, the specific activity of the [^{3}H]histidinol became only slightly higher than that of the NADH, suggesting that under these conditions the two compounds equilibrate a single hydrogen atom, namely, that transferred during the alcohol dehydrogenase step.

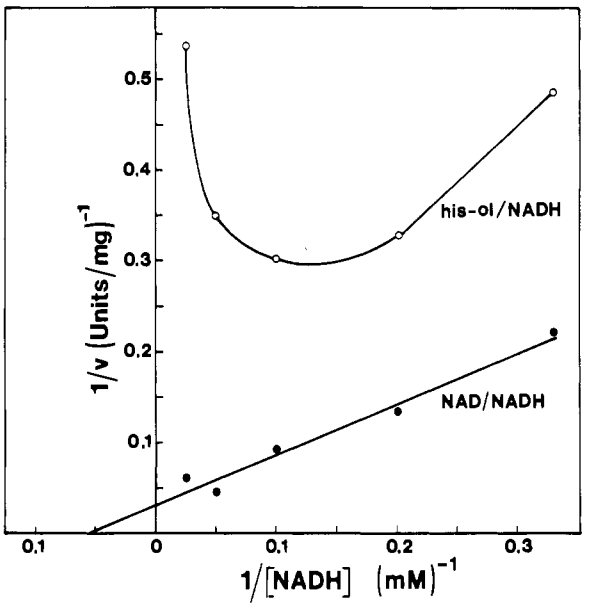


FIGURE 3: Kinetics of exchange reactions. Reaction mixtures (1 mL) contained 50 mM sodium glycinate, pH 9.2, 0.5 mM $MnCl_2$, 1 mM histidinol, and specified levels of NADH in a 2:1 ratio with NAD. For the NAD/NADH exchange (●) reactions contained 1×10^6 cpm of [4(S)-^{3}H]NADH and 2.8–26 μg of histidinol dehydrogenase. For the histidinol/NADH exchange (○) reactions contained 1×10^6 cpm of [4(R)-^{3}H]NADH and 5–25 μg of histidinol dehydrogenase. After 10 min of reaction at 30 °C, 100-μL samples were analyzed for exchange as described under Experimental Procedures.

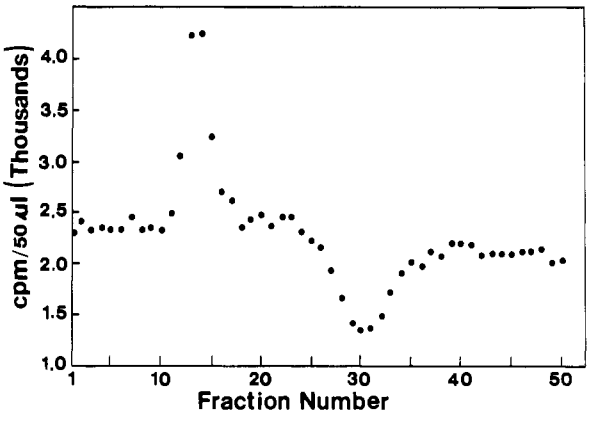


FIGURE 4: Hummel and Dreyer analysis of [^{3}H]histidinol binding. Experiments were performed at 10 μM [^{3}H]histidinol as described under Experimental Procedures.

When NAD and NADH concentrations were raised in constant (1:2) ratio (Figure 3), the rate of the NAD/NADH exchange increased in velocity following saturation kinetics. Under these conditions, the NAD/NADH exchange exhibited a V_{max} of 33 units/mg. In contrast, the histidinol/NADH exchange was severely inhibited by high concentrations of the NAD–NADH pair. This pattern was observed in several experiments. It was not possible to estimate a V_{max} for the reaction because of the curvilinear nature of the data. However, at low nucleotide concentrations, the rate of the histidinol/NADH exchange was 2–3-fold lower than that of the NAD/NADH exchange.

[^{3}H]Histidinol produced by the exchange reaction was used to measure histidinol binding to histidinol dehydrogenase. The technique of Hummel and Dreyer (1962) on the micro scale suggested by Fersht (1975) was employed. [^{32}P]P_i was used

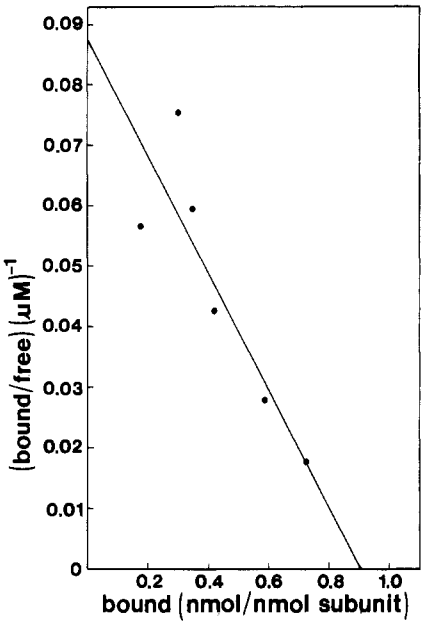


FIGURE 5: Scatchard-type plot of [^{3}H]histidinol binding to histidinol dehydrogenase. Data were obtained as described under Experimental Procedures.

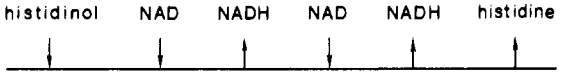
as an internal standard to measure the volume of individual drops. A typical data set at 10 μM histidinol is shown in Figure 4. The peak of radioactivity corresponding to bound histidinol was always well separated from the trough under the conditions used. The areas of the peak and trough were used to calculate the amount of histidinol binding. Results from a series of these experiments are shown replotted according to Scatchard (1949) in Figure 5. A K_D of 10 μM was estimated, with 1.7 sites per dimeric molecule (0.85 site/subunit). No evidence of cooperativity between binding sites was detected.

Similar experiments were conducted to measure [^{3}H]NAD and [^{3}H]NADH binding. In work not shown here, no binding was detected at 0.5 mM coenzyme and 0.2 mM subunit. Histamine (1 mM) did not allow for NAD or NADH binding. We estimate that we could have detected binding with a K_D of less than 10 mM with these techniques.

Histidinol dehydrogenase was found to interact weakly with Cibacron Blue substituted agarose, eluting in about 15 column volumes (not shown). In a series of experiments employing increasing concentrations of NAD, elution was progressively more rapid, occurring in 3 column volumes at 10 mM concentrations of the coenzyme. NADH gave similar results. Thus, the coenzymes are successful but weak eluting agents.

Discussion

Our interest in histidinol dehydrogenase is based on its ability to catalyze the sequential oxidation of an alcohol substrate and an aldehyde intermediate, apparently at a single active site (Adams, 1955). The exchange reactions and binding experiments reported in this paper allow us to understand the kinetics of the histidinol dehydrogenase reaction and to learn about the relationship between the two partial reactions. The results are best understood with reference to the kinetic mechanism postulated by Gorisch and co-workers (Gorisch, 1979; Burger & Gorisch, 1981) on the basis of steady-state measurements:



Substrate Binding. It is clear from the histidinol binding studies reported here that histidinol binds directly to two sites on the unliganded enzyme and the K_D (10 μM) is identical with the K_m measured under the same conditions. Thus, the measured binding must reflect normal binding to the catalytic site. Likewise, it is clear from similarly performed experiments that NAD binds poorly to the uncomplexed enzyme, although it also appears that histamine, which is an excellent competitive inhibitor vs. histidinol does not permit NAD or NADH binding. Results with Cibacron Blue–agarose suggest that some binding of NAD and NADH to the unliganded enzyme must occur, but with low affinity. The results indicate that binding of the first two reactants is highly ordered, with histidinol leading. The proposed kinetic order of binding is unusual for dehydrogenases, in that NAD generally binds first, causing an enzyme conformational change (Grau, 1982). Other exceptions include UDP-glucose dehydrogenase (Ordman & Kirkwood, 1977) and possibly 3-hydroxy-3-methylglutaryl-CoA reductase (Rogers et al., 1983), which are the other two known four-electron dehydrogenases. In addition, yeast aldehyde dehydrogenase exhibits a substrate-leading order (Bradbury & Jakoby, 1971). For glutamate and liver alcohol dehydrogenase random and "preferred" NAD-leading kinetic pathways have been demonstrated (Dalziel, 1975).

A second line of evidence for the reaction order comes from the kinetics of the exchange reactions, which are discussed more fully in the following section. The inhibition of the histidinol/NADH exchange at high NAD and NADH concentration, an inhibition not shared by the NAD/NADH exchange, indicates that at high nucleotide concentration the labeled histidinol produced via the exchange cannot dissociate from the enzyme. The only reasonable explanation for this failure to dissociate is that the histidinol does not dissociate from the ternary enzyme–histidinol–NAD complex but only from the enzyme–histidinol complex, whose concentration is low in the presence of high nucleotide level. Inhibition of exchange reactions is a classical evidence for diagnosis of an ordered mechanism (Purich & Allison, 1980).

The First Oxidation. Two lines of evidence suggest that the histidinol/NADH exchange studied here arises solely from reversal of the first partial reaction. First, measurements during exchange show that the specific activity of histidinol rises only to that of NADH. It is known from work on a fungal histidinol dehydrogenase (Davies et al., 1972) that both reductions of NAD go via *R* stereochemistry. We have recently shown (this laboratory, unpublished results) that this stereochemistry is preserved in the *Salmonella* enzyme. Thus, if both partial reactions contributed to the exchange, then histidinol containing two NADH hydrogens should be found, and specific activity of the substrate should be twice that of NADH. Second, in NMR studies not shown here, we have found that the histidinol/NADH exchange results in the stereospecific labeling of a single histidinol hydrogen (whose absolute stereochemistry is unknown). Thus, the existence of the histidinol/NADH exchange demonstrates the reversibility of the first oxidation. It has been shown previously that histidinol dehydrogenase could catalyze the reduction of the highly unstable histidinaldehyde to histidinol (Adams, 1955; Gorisch & Holke, 1985) at rates approaching that of forward net catalysis, but since histidinaldehyde does not dissociate from the active site during overall catalysis (Adams, 1955), the kinetic relevance of using externally added aldehyde is not clear.

It is more problematic to determine unequivocally the origin of the NAD/NADH exchange. It is most likely that the sole

origin is a reversal of the first oxidation step since only one atom of ^3H from [4(R)-^3H]NADH is incorporated into exchanged histidinol. If any part of the NAD/NADH exchange can arise from reversibility of the second oxidation, it thus must operate on a form of histidinaldehyde intermediate that lacks a kinetically reversible connection to histidinol. Since both possible NAD/NADH exchange reactions must involve the enzyme–histidinaldehyde intermediate, the required separation of the two NAD/NADH exchange reactions would necessitate two separate enzyme–histidinaldehyde complexes. We regard this as unlikely.

The rates of the two exchange reactions are quite high, with the NAD/NADH exchange capable of rates substantially higher than V_{max} for net catalysis (10–15 units/mg). Since exchange reactions were carried out under conditions of NADH concentration (2:1 NADH:NAD in Figure 3) that are quite inhibitory to net catalysis [the K_m for NAD is 1 mM, while the K_I for NADH is 0.3 mM (Burger & Gorisch, 1981)], it seems that the actual catalytic steps in the exchange reactions must be capable of velocities well above that of net catalysis. Preliminary investigations of kinetic isotope effects in the two partial reactions also indicate that the first oxidation reaction does not participate in rate limitation of the overall reaction of histidine production (unpublished results). The lower rate of the histidinol/NADH exchange could result from either of two sources: the release of bound histidinol could be slow, or the transfer of a hydrogen from labeled NADH could become partially rate limiting when slowed by a tritium isotope effect (since no tritium–carbon bond is broken in the NAD/NADH exchange reaction, there is no primary isotope effect). The kinetics of the histidinol/NADH exchange suggests that the first possibility may be correct: at high concentrations of NAD + NADH, a slowly dissociating enzyme–histidinol complex would partition toward NAD binding, with consequent inhibition of exchange.

The Irreversible Step. Measurements with histidine have suggested that the net reaction of histidinol dehydrogenase is irreversible. However, the mechanistic origin of this irreversibility is unknown. The lack of a histidine-supported NAD/NADH exchange shows clearly that free histidine and NADH cannot reverse to the step of NAD release. The irreversible step could thus be histidine release, which is not likely as histidine can bind to the enzyme as a competitive inhibitor vs. histidinol (Burger & Gorisch, 1981). Alternatively, the second oxidation step, or the hydrolysis of a covalent (e.g., thiol ester) derivative of histidine at the active site, might be irreversible. A choice between the latter two possibilities cannot be made without further knowledge of the redox mechanism involved in this two-step reaction. Although the current work shows that the second oxidation does not reverse under exchange conditions to produce ditritiohistidinol, this may be a consequence of close kinetic coupling between NADH release and ester hydrolysis, rather than an indication of an irreversible redox step.

Registry No. NAD, 53-84-9; NADH, 58-68-4; histidinol dehydrogenase, 9028-27-7; histidinol, 501-28-0.

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